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Angiogenesis, formation of new blood vessels, plays a key role in breast cancer growth by providing a method for hematogenous spread of the tumor, as well as providing nutrients for tumor metastasis. Rational treatment strategies for breast cancer must take into account the molecular mechanisms by which cancer develops, maintain its growth and finally spread to other parts of the body. The pleiotrophin (PTN) signaling pathway is known to be important in angiogenesis and breast cancer growth, but the exact mechanism by which PTN acts has not been elucidated. Recently, we identified a cell-surface receptor tyrosine kinase, anaplastic lymphoma kinase (ALK) receptor, as a receptor for PTN. In the previous report, we demonstrate the biological relevance of pleiotrophin signaling via ALK by targeting ALK with hammerhead ribozymes. In this report, we will demonstrate the role of the PTN/ALK interaction in tumor development and proliferation by overexpressing ALK dominant-negatives to disrupt PTN/ALK signaling.						
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### INTRODUTION

Angiogenesis plays key roles in breast cancer by providing essential nutrients for tumor growth, and by providing access to the general circulation for tumor metastasis (1). Signaling pathways employed by well known angiogenic factors, such as basic fibroblast growth factor (bFGF), have been well defined (2). The signaling pathway of pleiotrophin (PTN) has also been shown to be important in angiogenesis and breast cancer growth, although the exact mechanism by which PTN acts has not been elucidated. Recently, we identified a receptor tyrosine kinase, anaplastic lymphoma kinase (ALK), as a receptor for PTN (3). Here we report the role of PTN/ALK interaction in tumor development and proliferation by over-expressing ALK dominant-negatives to disrupt PTN/ALK signaling.

### **BODY**

The goal of Aim 2 was to use ALK dominant-negative mutants to determine the role of PTN/ALK in tumor development and proliferation. The first step towards this goal was to construct dominant-negative ALK mutants. ALK is a receptor tyrosine kinase consists of an extracellular domain (ECD), a transmembrane domain (TM), and an intracellular kinase domain (KD) (Figure 1a). Assuming that the ECD and the transmembrane domain are sufficient for ligand binding and receptor dimerization, the construction of a mutant lacking KD should act as a dominant-negative. Two dominant-negative mutants were created: an ALK-ECD mutant, which codes amino acid 1-980 in the ECD, and an ALK-TM mutant, which codes amino acid 1-1080 (ECD and TM domain) (Figure 1a and 1b). Both ALK-ECD and ALK-TM dominant-negative mutants were constructed by inserting the ECD coding region (nucleotides 1-3088), or ECD plus the TM coding region (nucleotides 1-3386) (Figure 1b), into a commercially available

mammalian expression vector, pcDNA3.1 *My*c-His (Invitrogen). The mutant constructs were verified both by enzyme digestions and sequencing of the insertion sites (Data not shown).

The second part of this aim is to stably transfect the ALK-ECD mutant and the ALK-TM mutant into human cancer cell lines. As mentioned in last year report, there is a lack of good antibody towards ALK; therefore RNase protection assay is employed to detect endogenous expression of ALK. U87MG (a human glioblastoma cell line) is used for stable transfection of ALK dominant-negative mutants because their endogenous expression of wild-type ALK mRNA could be more easily determined through RNase protection assay. Stable mutant transfectants in U87MG cells were confirmed using western blot analysis with anti-myc antibody (figure 2). As expected, ALK-ECD mutant is detected by western blots in the condition media (figure 2), but not in the cell lysate of the ALK-ECD transfected cells. This is due to that fact that this mutant lacks the transmembrane domain it requires to anchor it to the cell membrane, thus the ALK-ECD transfected cells secrete it. In contrast, the ALK-TM mutant contains the transmembrane domain, therefore it found on the cell membrane (figure 2) and not in the condition media of ALK-TM transfected cells. Both of these mutants should act as dominant-negative by sequestering and dimerizing with the endogenous wild-type ALK, thereby inhibiting the kinase activity of ALK.

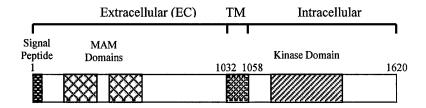
The final task of Aim 2 was to determine the biological relevance ALK dominant-negative mutants on PTN/ALK signaling. A frequent characteristic of early neoplastic transformation of epithelial cells is growth in with the loss of strong cell-cell adhesion and adhesion to the basement membrane, resulting in anchorage-independent growth. The soft agar assay is a very sensitive method for detecting alterations in the growth requirements of tumor cell lines. A single cell suspension of each cell line was plated in agar and allowed to grow for

two weeks, at which time those cells capable of growing in the absence of contact with a basement membrane would have formed colonies that could be detected by our image analysis equipment. This experiment was carried out in empty vector (pcDNA) transfected or ALK-ECD transfected cells, plated with different doses of PTN. While ALK-ECD transfected cells formed less colonies than pcDNA transfected cells at higher doses of PTN, there was no significant difference (Figure 3). Similar experiments were carried out using ALK-TM transfected versus pcDNA transfected cells, which yielded the same result (data not shown). To determine whether the presence of ALK-ECD or ALK-TM will affect the proliferation rate of these cells, cells were plated with different doses of PTN for 3 days, then WST-1 proliferation assay (a colorimetric assay for the quantification of cell proliferation based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases) was performed (Figure 4). The data indicates that at higher concentration of PTN, although the presence of ALK-ECD reduces the rate of proliferation, the presence of ALK-TM does not (Figure 4).

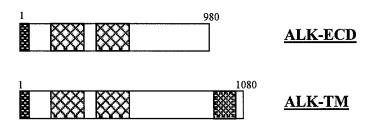
# FIGURE 1

A.

### Anaplastic Lymphoma Kinase (ALK) or PTNR



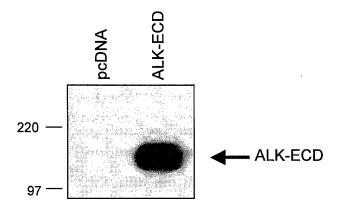
**B.** 



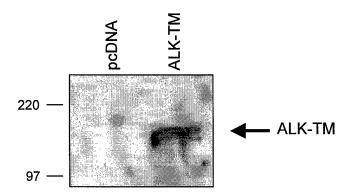
**FIGURE 1: Construction of ALK dominant-negative mutants.** A. A diagram illustrating the domains of ALK. B. A schematic of the illustrating the portions of ALK inserted into a mammalian expression vector. ALK-ECD represents 1-980 amino acids while ALK-TM represents 1-1090 amino acids.

## FIGURE 2

 $\boldsymbol{A}$ 



**B**.



**Figure 2:** Expression of ALK dominant-negatives in U87 MG cells. Both the condition media and cell lysates of ALK-ECD and ALK-TM transfected cells were run on SDS-PAGE gels, transferred onto PDVF membrane and western blotted with anti-myc antibody. *A.* ALK-ECD is only detected in the condition media of ALK-ECD transfected cells. *B.* ALK-TM is only detected in the cell lysates of ALK-TM transfected cells. The expected molecular weight of ALK-ECD and ALK-TM are 111 kDa and 122 kDa, respectively. The apparent molecular weight is higher due to high glycosylation of the protein.

# Figure 3

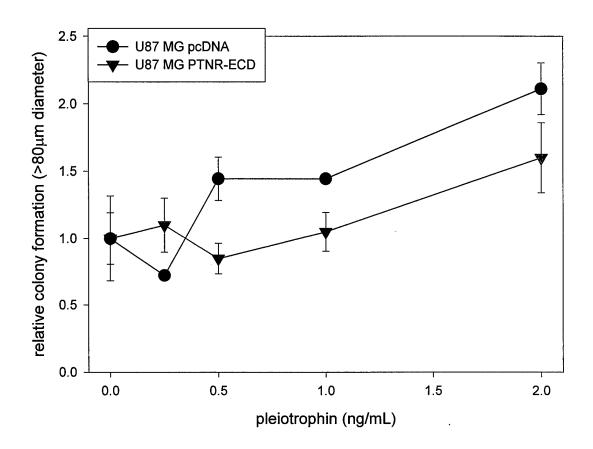


FIGURE 3: Expression of ALK-ECD mutant in U87MG cells does not significantly reduce colony formation in soft agar. Soft agar colony formation with the wild-type U87MG cells (●) or with ALK-ECD transfected cells (▼). The expression of ALK-ECD does not significantly alter soft agar colony formation.

## Figure 4

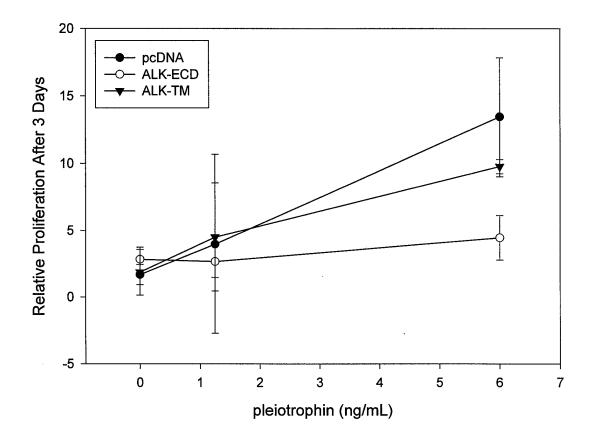


FIGURE 4: Expression of dominant-negative ALK mutants does not significantly reduce proliferation of U87MG cells. Wild-type U87MG cells (●), ALK-ECD transfected cells (○) or ALK-TM transfected cells (▼) were plated in 96 wells plates for three days before WST-1 proliferation assay was performed. The presence of dominant-negative ALK mutants in U87MG cells does not significantly alter proliferation of U87MG cells.

### **KEY RESEARCH ACCOMPLISHMENTS**

- Dominant-negative mutants of ALK were constructed by excision of the ECD (1-980aa) or ECD plus TM (1-1088aa) of ALK, and inserting them into an INVITROGEN mammalian expression vector containing a Myc/His tag (Figure 1).
- Stable transfectants expressing ALK-ECD and ALK TM were generated and their expression was determined by western blot, using an anti-myc monoclonal antibody (Figure 2)
- Soft agar assays were used to detect changes in phenotype of ALK-ECD transfected cells (Figure 3).
- WST-1 proliferation assays were used to detect changes in the biological activity of ALK-ECD and ALK-TM transfected cells (Figure 4).

### REPORTABLE OUTCOMES

### **Publications:**

Stoica GE\*, <u>Kuo, A</u>\* (\*co-first authors), Aigner A, Sunitha I, Souttou B, Malerczyk C, Caughey DJ, Wen D, Karavanov A, Riegel AT, Wellstein A. (2001) *Identification of Anaplastic Lymphoma Kinase as a receptor for the growth factor pleiotrophin.* J. Biol. Chem., 279 (18): 16772-16779.

#### Abstracts:

**Kuo AH**, Stoica GE, Powers C, Bowden E, Riegel AT, Wellstein A. (2001) *Characterization of pleiotrophin signal transduction through a receptor tyrosine kinase*. 92<sup>nd</sup> Annual Meeting, American Association for Cancer Research.

Stoica GE, Aigner A, Powers C, List H-J, Kuo A, Bowden ET, Riegel AT, Wellstein A. (2001)

Pleiotrophin binds and activates a receptor tyrosine kinase. 92<sup>nd</sup> Annual Meeting, American Association for Cancer Research.

#### **CONCLUSIONS**

While the above described experiments did not yield significant results, they did meet the goals described in Task 2 of the approved grant application. From the previous annual report and publication, ALK is detected in U87MG cells (4). U87MG cells are also known to express a variety of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) (5). Perhaps the expression of the dominant-negative constructs of ALK (i.e. ALK-ECD and ALK-TM) was not high enough in these cells, thereby unable to sequester most of the endogenous ALK. This would result in non-significant reduction in soft-agar growth and cell proliferation (Figure 3 and 4). Alternatively, other signaling pathways in U87MG cells might compensate inhibition of PTN signaling through ALK by ALK dominant-negatives. Another possible explanation is that PTN signaling through ALK activates a survival pathway, as opposed to a proliferative pathway. Both colony formation in soft agar and WST-1 proliferation assay may not be the best experiments to detect such a pathway. The development of immunoprecipitaion and western blot is underway to detect proteins that are activated in survival pathways.

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